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14. ABSTRACT

The project was to determine how the tumor suppressors ELL-associated factors 1&2 (EAF1 and EAF2) are regulated in the prostate and the functions of EAF1 and EAF2 by examining an observed protein modification ~50 kDa heavier than the predicted weight of EAF1 and EAF2 and to determine how sumoylation and protein degradation affect EAF1 and EAF2 sub-cellular localization. Work on specific aim 1, identifying the modification, discovered that the modification was most likely a homodimerization that can be observed under denaturing conditions. Mutating the potential sumoylation sites did not affect modification. It was determined by the thesis committee that finishing the EAF1 and EAF2 modification project would take longer than the allotted grant time. The recommendation is to switch to a project examining how EAF2 interacts with FOXA1 and HMGB1. The new project pursues the same issues, EAF1 and EAF2 function and regulation, from a different angle

15. SUBJECT TERMS
prostate, prostate cancer, EAF1, EAF2, tumor suppressor, protein modification, protein regulation, FOXA1, HMGB1

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Introduction

Prostate cancer is the most commonly diagnosed cancer and the second leading cause of cancer death in men in the US (*1*). Prostate carcinoma results from the accumulation of mutations in prostatic glandular epithelial cells. This often occurs because the mutations alter the normal pathways of protein regulation, increasing or decreasing the levels of key regulatory proteins or their activity. Frequently, protein levels are regulated through the addition or removal of covalently attached protein modification. Understanding how tumor suppressors are regulated at the protein level is important to devising therapeutic and/or preventive strategies. ELL-associated factor 2, also known as androgen up-regulated 19, (EAF2/U19) is a putative transcription factor and was discovered to be a protein up-regulated by the androgen receptor in the normal prostate, but down-regulated in prostate tumors (*2*). Over-expression of EAF2 induces apoptosis in prostate cancer cell lines while EAF2 knockout in mice resulted in lung cancer, liver cancer, lymphoma, as well as prostatic intraepithelial neoplasia, the putative precursor of prostate cancer. ELL-associated factor 1 (EAF1) is the only homolog of EAF2 in mammalians and is believed to play a role in leukemia (*3, 4*). EAF1 and EAF2 share 74% identity at the amino acid level. EAF1 becomes delocalized from the nucleus in acute myeloid leukemia due to a chromosomal rearrangement, inactivating EAF1(*4*).

Although these two proteins play a role in tumorigenesis, the mechanism and regulation pathways are unknown. Preliminary data from Western blots showed the presence of modified EAF1 and EAF2, which are ~50 kDa heavier than the unmodified EAF1 and EAF2. Furthermore, preliminary data showed that the addition of MG132 increases EAF1 and EAF2 protein levels and causes EAF2 to localize to the nucleus. According to a computer analysis using SUMOplot (*5*), there are also 2 potential sumoylation sites in EAF1, K54 and K174, as well as two potential sumoylation sites in EAF2, K18 and K164. Increased sumoylation causes a drop in EAF1 levels and a shift in EAF1 localization. We hypothesized that EAF2 and EAF1 function is affected by this protein modification which might in turn modulate their potential roles as tumor suppressors in the prostate. This research will give a better understanding of how this family of proteins is regulated and the specific role they play in prostate cell proliferation, survival, differentiation.

The three specific aims for the project were as follows: specific aim 1 was to determine the nature of the modification of EAF1 and EAF2, specific aim 2 was to determine if the modification affects EAF1 and EAF2 function, and specific aim 3 was to understand the roles of sumoylation and protein degradation in EAF1 and EAF2 localization. For specific aim 1, 18 months were allotted and divided as follows: identifying the modification using mass-spectrometry and biochemical techniques (months 1-10), generating EAF1 and EAF2 mutants (months 11-18), studying the effect of the modification on protein levels and localization (months 1-18), and submitting a paper (month 18). For specific aim 2, 9 months were allotted and divided as follows: performing co-immunoprecipitations to identify effects of modification on protein interactions (months 19 – 24) and experiments to determine if the modification affects cell growth and survival (months 25-28). For specific aim 3, 7 months were allotted and divided as follows: determining the roles of sumoylation and ubiquitination in EAF1 and EAF2 sub-cellular localization (months 29 – 36) and submit a manuscript detailing the functional effects of the modification on EAF1 and EAF2 association with binding partners and on cell growth and survival (month 36).

Body

When the grant was submitted for the project, it was thought that the observed modification (Figure 1) was due to polyubiquitination or polysumoylation. Right after the grant was funded an experiment was performed to test the possibility that the modification was a homozygous dimerization. The experiment, as it is shown in Figure 2, revealed that the modification was a homozygous dimerization, not polyubiquitination or polysumoylation. Cells from the HEK 293 (293) cell line were transfected with GFP-EAF1 and HA-EAF1 and then the cell lysates were immunoprecipitated with rat anti-GFP agarose beads and then the samples were denatured before blotting. It is also a homozygous dimerization that can be observed after denaturation, which means the dimerization requires very strong binding, likely a covalent bond.

When homodimers are observed for transfected proteins, there is a possibility that the dimerization is due to protein aggregation. Protein aggregation can occur when a protein is over-expressed. So work was started to determine if the homodimer was occurring in stably transfected cell lines and endogenously. Then issues started occurring with the variability of the dimer. In some blots, like in figure 1, there is less dimer than monomer. In other blots, like Figure 2A, there is more dimer than monomer. In other blots, like Figure 2B, the monomer is observed but not the dimer. In another set of blots not shown, the dimer is present but not the monomer. Now the project was looking at a covalent homodimerization that is affected by some unknown variable that alters the ratio of monomer to dimer. Experiments were started to try and determine which exons were required for the dimerization to occur, but the variability of the dimerization made it difficult to reach a conclusion and the experiments were halted at the recommendation of the thesis committee.

The mutants to be developed in specific aim 1 were mutants that had the potential sumoylation and ubiquitination sites mutated so the sites cannot be sumoylated or ubiquitinated. The sites for EAF1 are lysine (K) 54 and K174. The plan was to mutate them to arginine (R) using PCR-based cloning. EAF1 K54R and EAF1 K174R were made but had no effect on the homodimer as seen in Figure 3. Mutant making was not carried out further after the homodimerization was discovered.

After discussing the discovery that the modification is actually a homodimerization and occurs at variable protein levels with the PI's thesis committee, the recommendation was made to switch projects. It would take longer than two years to gather enough evidence to convince other scientists that an endogenous covalent homodimer was being formed by EAF1 and then would still have to do all of the functional experiments. Due to the difficulties with the homodimerization project, work was started on a side project. An RNAi screen in *C. elegans* worms had been started to find proteins that interacted with the *C. elegans* ortholog to EAF1 and EAF2/U19, *eaf-1*. The screen was performed by treating *eaf-1*KO worms with RNAi and finding RNAi that worsened the phenotype of the *eaf-1*KO worm. When *eaf-1* is knocked out in *C. elegans*, the worms produce fewer offspring than wild-type worms. The screen found two genes, *hmg-1.2* and *pha-4*, that when they are knocked down by RNAi in *eaf-1*KO the worms are sterile as shown in Figure 4. The side project was to determine if the human ortholog of *hmg-1.2*, HMGB1 and the human ortholog of *pha-4*, FOXA1 interact with EAF1 and EAF2/U19. Like the original project, the new project would elucidate how EAF1 and EAF2 function and how EAF1 and EAF2 are regulated. The current focus is on EAF2 and eventually the focus will shift back to EAF1.

FOXA1 is forkhead box protein A1. FOXA1 is a pioneer factor for the androgen receptor (AR). FOXA1 binds to chromatin, rearranges the nucleosomes to make open chromatin, and then AR binds to FOXA1 to begin gene transcription (*6, 7*). FOXA1 is required for the development of ductal epithelial cells in the prostate (*8*) Also, FOXA1 protein levels are elevated in metastatic and castration-resistant compared to normal prostate tissue and primary prostate cancer (*9*). Co-transfections of FOXA1 with EAF1 and EAF2/U19 were performed. As seen in Figure 5, FOXA1 protein levels were elevated in the presence of EAF1 and EAF2, while EAF1 and EAF2 protein levels were reduced. Work is underway to replicate the initial observation. Furthermore, plasmid dosage assays, where 1 µg of FOXA1 and a varying amount of EAF2/U19 complimented to 2 µg of DNA by empty vector or where 1 µg of EAF2/U19 and a varying amount of FOXA1 complimented to 2µg of DNA by empty vector are transfected into PC3 cells. The goal is to determine if EAF2 alters FOXA1 protein levels in a dose-dependent manner and if FOXA1 alters EAF2 protein levels in a dose dependent manner. Cycloheximide assays will be performed to see if EAF2 alters the stability of FOXA1 and if FOXA1 alters the stability of EAF2. After performing the cycloheximide assays, the plan is to perform pulse-chase assays. The pulse-chase assays will reveal if the stability of EAF2 or FOXA1 is altered when transcription is occurring. After the pulse-chase assays, it is planned to do a luciferase assay using the probasin promoter to test if EAF2 affects FOXA1-dependent transcription. It is also planned to test if the combination affects cell survival by colony-formation assays, BRDU assay, and by measuring the levels of caspase 8.

HMGB1 is high mobility group box 1 protein. It is a non-histone non-specific DNA binding protein in the nucleus, a mediator of autophagy in the cytoplasm, and a cytokine when it is extracellular (*10*). High levels of HMGB1 are associated with worse outcomes in prostate cancer (*11*). When *eaf-1* and *hmg-1.2* were co-transfected in 293 cells, *hmg-1.2* shifted from the nucleus to the cytoplasm as seen in Figure 6A. The shift was statistically significant. When HMGB1 was co-transfected with EAF2/U19, the same trend was observed in Figure 6B. By t-test, the means of the GFP-HMGB1 +RFP and GFP-HMGB1+RFP-U19/EAF2 N>C are significantly different as are the means for the N=C, $p<0.05$ for both. The variances are not significantly different. More replications are needed to test if the variances are also different. Co-immunoprecipitations were performed to determine if HMGB1 interacts with EAF1 and EAF2 by direct protein interactions. Binding of HMGb1 to EAF1 and EAF2 was not detected by co-immunopreciptiation in figure 6. Work is also underway to determine if HMGB1 expression prevents EAF2/U19 mediated apoptosis. This is being done by performing colony formation assays to determine if HMGB1 alters cell-death when co-transfected with EAF2. The levels of caspase 8 will also be measured in cells co-transfected with HMGB1+ EAF2 or HMGB1+empty vector or EAF2+empty vector by western blot.

If the project change is authorized, the new specific aims would be specific aim 1: determine if EAF2 and FOXA1 interact at a molecular level and if they affect cell growth and survival; specific aim 2: determine if EAF2 and HMGB1 interact at a molecular level and if they affect cell growth and survival. Testing if EAF1 also interacts with FOXA1 and HMGB1 will be a future project. While the project is changing, it should still accomplish the original goal, which was to determine the functions and regulation mechanisms of EAF2, a prostate cancer tumor suppressor.

Through this project, the PI has continued gaining skills necessary to make her a better independent researcher. These skills, such as improving experimental design, planning, technique and troubleshooting are the basic traits of all independent researchers. Additionally, by presenting at the Society for Basic Urology Research annual conference and at departmental meetings she has learned how to better present data to peers who are not intimately acquanted with her project.

Key Research Accomplishments

- The observed modification that started the project was revealed to be a covalent dimerization after several co-immunoprecipitations.

Reportable Outcomes

- Presented a poster at Society for Basic Urology Research (SBUR) on research

Conclusion

The original project was to determine the nature of the observed modification of EAF1 and EAF2, determine if the modification affects EAF1 and EAF2 function, and to understand how sumoylation and protein degradation affect EAF1 and EAF2 sub-cellular localization. EAF1 is a potential tumor suppressor in prostate cancer and EAF2 is an androgen-receptor regulated tumor suppressor in prostate cancer. Work on aim 1, determining the nature of the modification, revealed the modification was not polyubiquitination or polysumoylation as initially suspected, but was likely instead a covalent homodimerization. Due to the variability of the dimerization, as well as the difficulty in producing convincing evidence that a covalent dimerization was occurring in a physiologically relevant manner, it is recommended instead to shift focus to the project focused on the interactions of FOXA1 and HMGB1 with EAF2. While approaching the problem from a different angle, the project has the same basic goals of better understanding the functional and regulatory pathways of this tumor suppressor. Having a better understanding of how this tumor suppressor family functions and how it is regulated will provide targets for prostate cancer therapy in the future.

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Appendix A: Figures

Figure 1

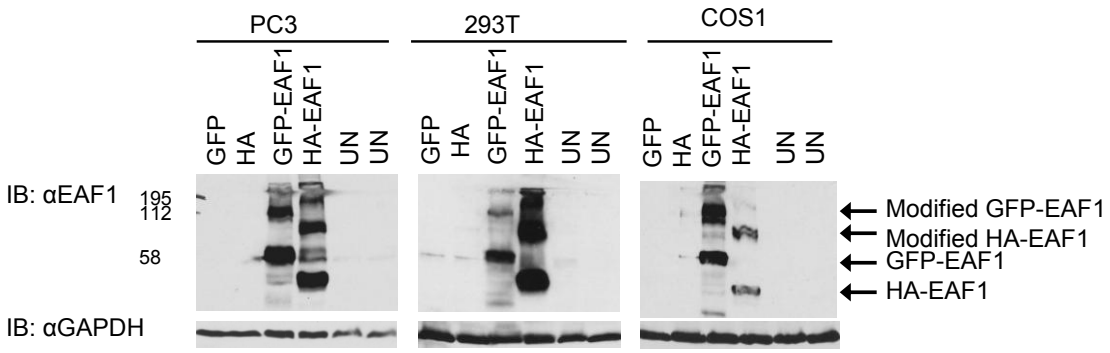
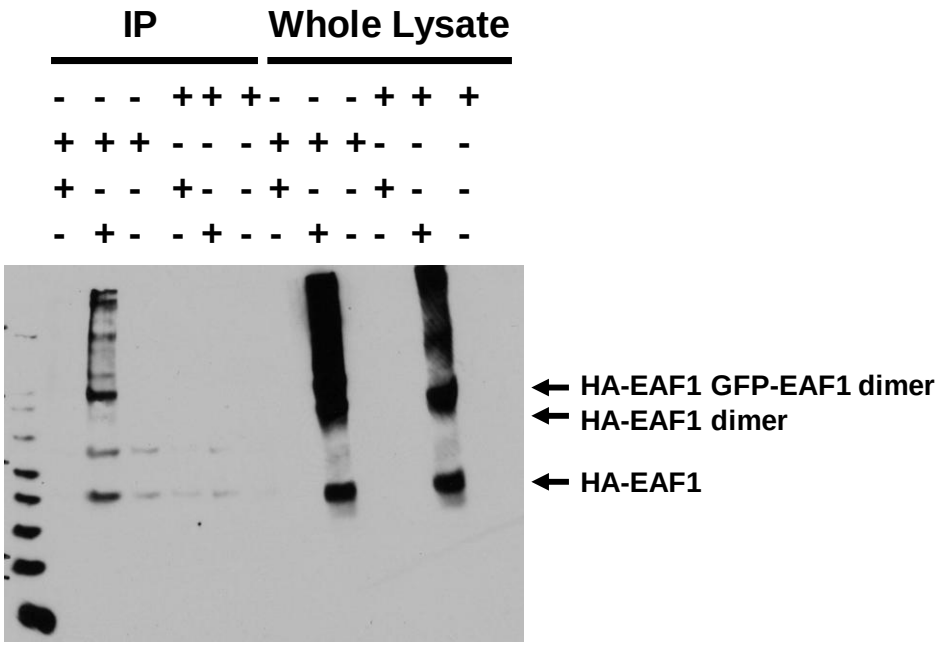
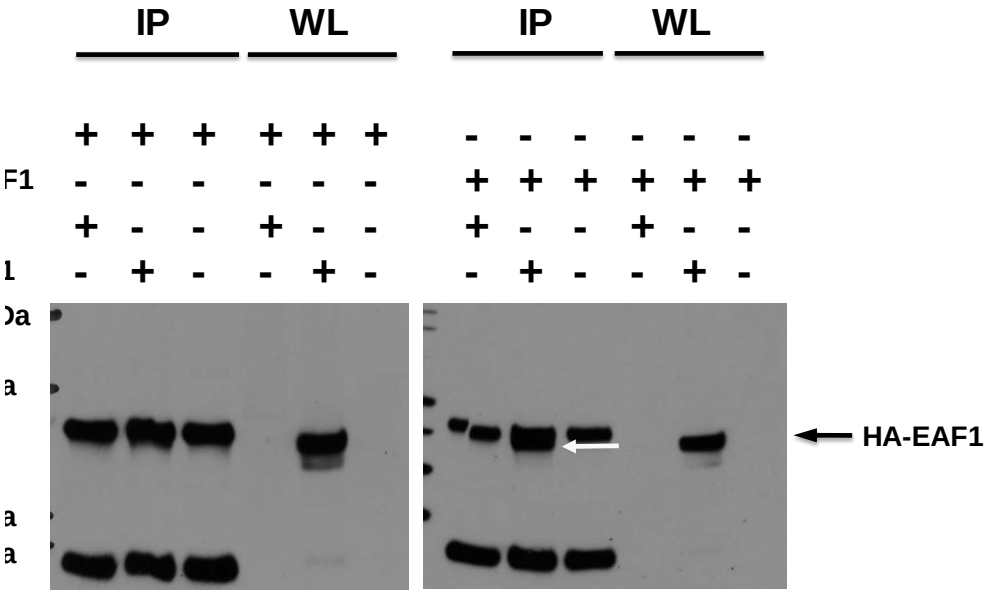


Figure 1 Modification observed in PC3, 293T, and COS1 cells. Observed in both HA and GFP tagged EAF1. Probed using anti-EAF1 antibody (Santa Cruz sc-67624)





nsfection of GFP-EAF1 and HA-EAF1 in COS1 cells. Homodimer can be
aturing conditions. B) IP = immunoprecipitate. WL = whole lysate. HA-EAF1
›-EAF1 is immunoprecipitated, revealing the higher weight band is a

Figure 3

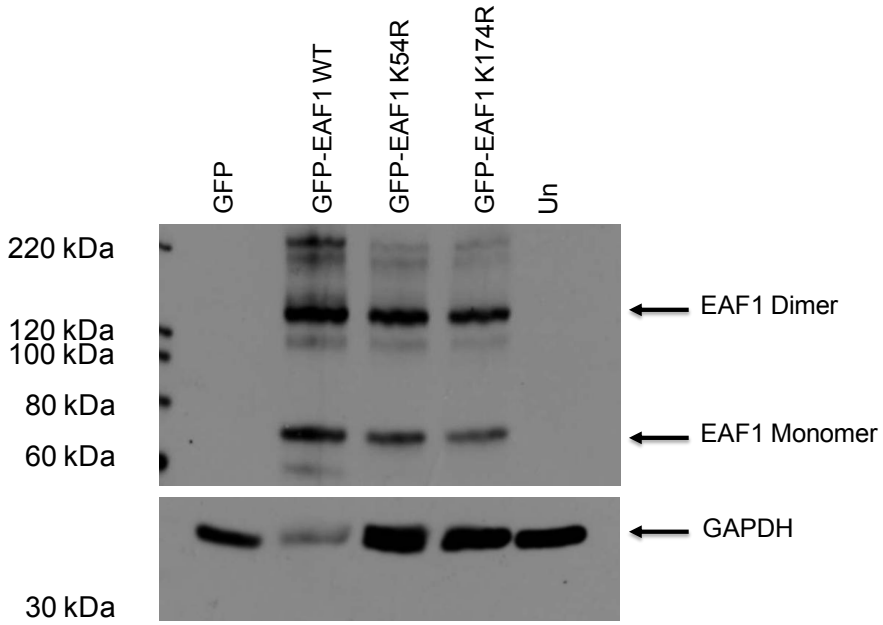


Figure 3 Mutating the potential sumoylation sites did not inhibit dimerization. It did reduce the amount of EAF1 protein observed.

Figure 4

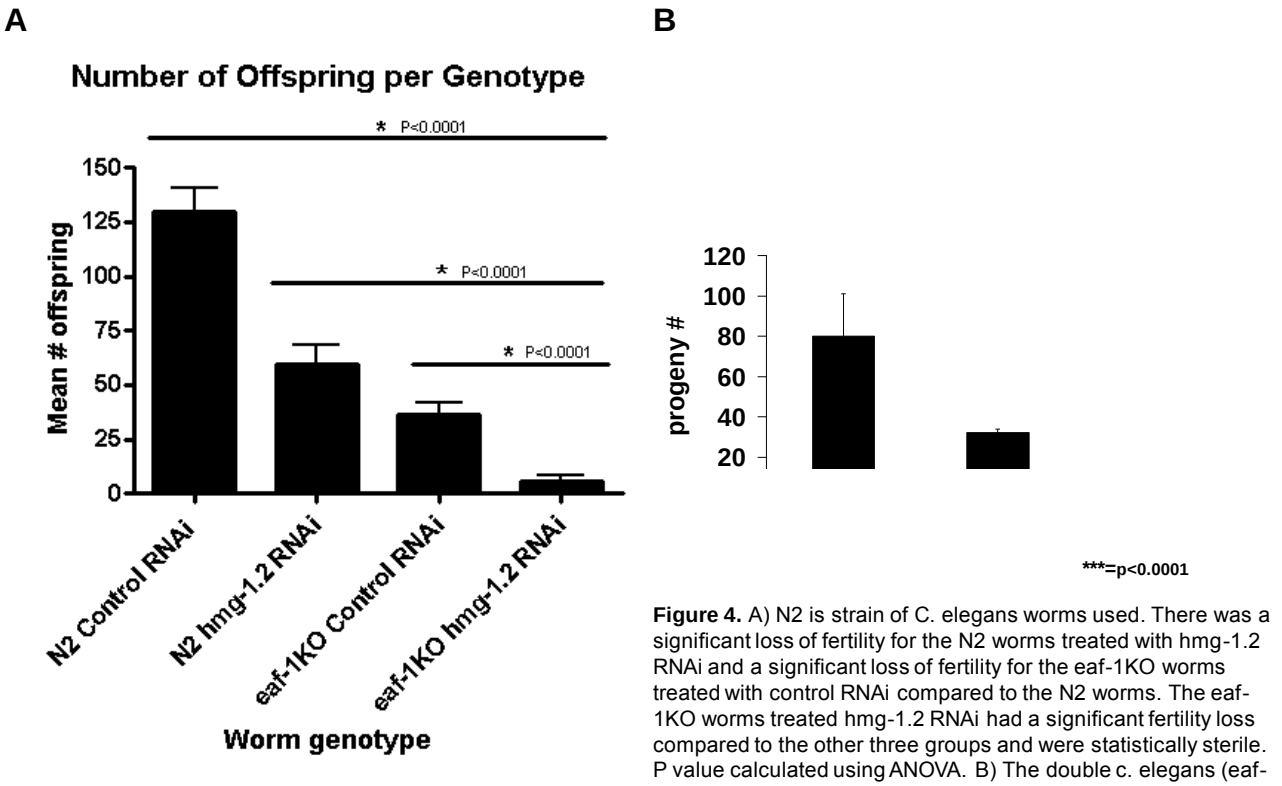


Figure 4. A) N2 is strain of C. elegans worms used. There was a significant loss of fertility for the N2 worms treated with hmg-1.2 RNAi and a significant loss of fertility for the eaf-1KO worms treated with control RNAi compared to the N2 worms. The eaf-1KO worms treated hmg-1.2 RNAi had a significant fertility loss compared to the other three groups and were statistically sterile. P value calculated using ANOVA. B) The double c. elegans (eaf-1KO worms treated with pha-4 RNAi) were sterile and the loss of fertility was significant. P value calculated by ANOVA.

Figure 5

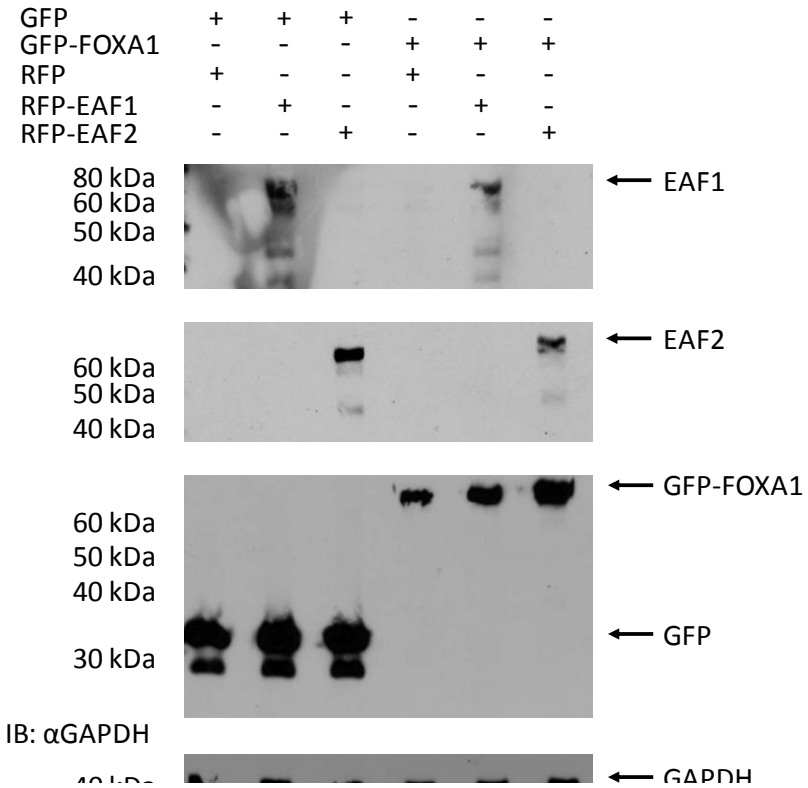


Figure 5 GFP-FOXA1 was co-transfected with RFP-EAF1 and RFP-EAF2EAF2. EAF1 protein levels were reduced in the presence of FOXA1. EAF2 protein levels were reduced in the presence of FOXA1. FOXA1 protein levels were increased in the presence of EAF1 and EAF2.

Figure 6

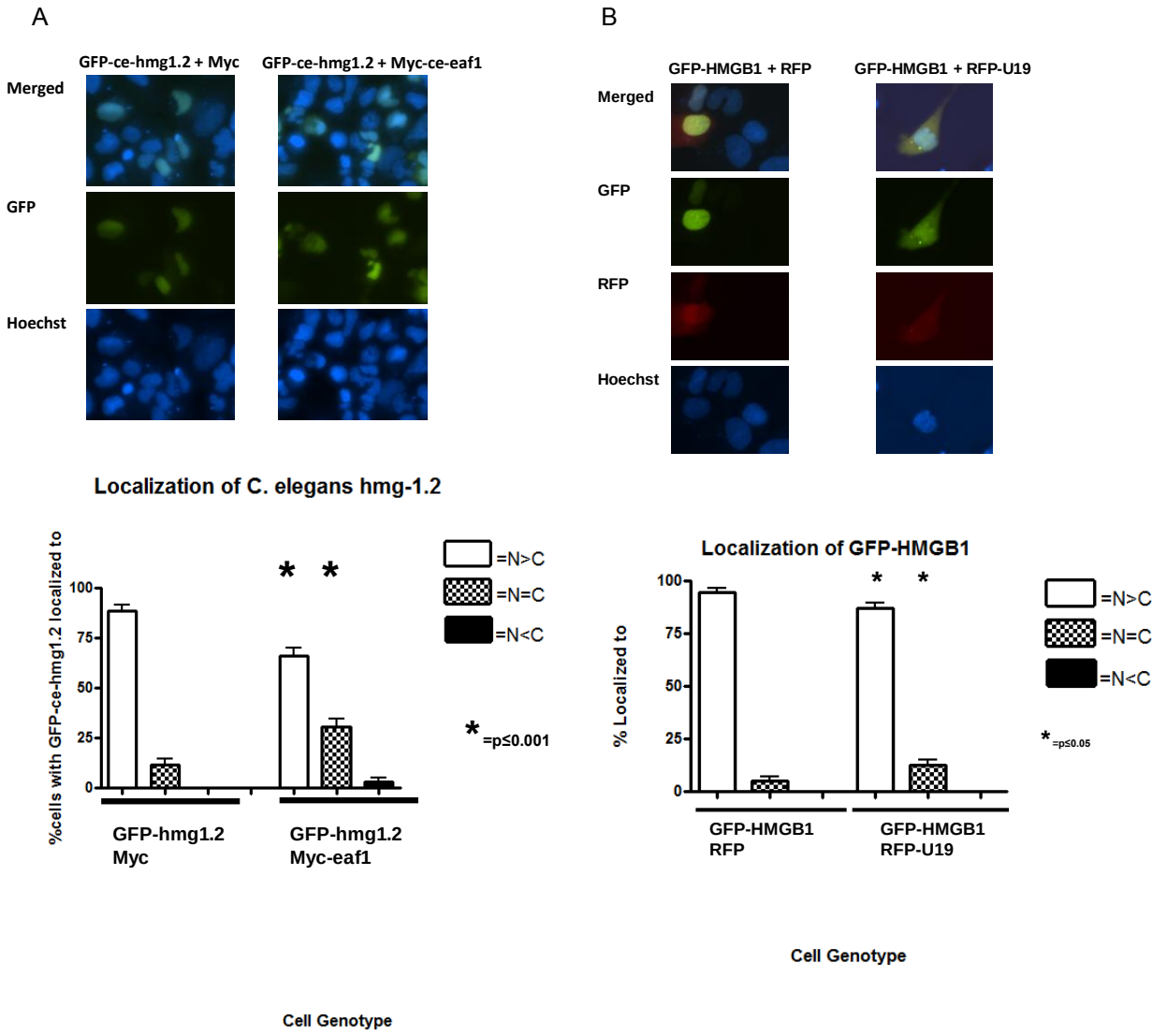


Figure 6 N>C is GFP expressed stronger in the nucleus than the cytoplasm, N=C is GFP expressed equally in both nucleus and cytoplasm, N<C is GFP expressed stronger in the cytoplasm than the nucleus. A) Statistically significant movement of GFP-hmg1.2 from nucleus to cytoplasm when co-transfected with myc-eaf-1 compared to GFP-hmg1.2 co-transfected with empty vector. Statistics calculated using ANOVA. B) Statistically significant movement of GFP-HMGB1 from nucleus to cytoplasm when GFP-HMGB1 is co-transfected with RFP-U19/EAF2. Statistics calculated by t-test. Means were significantly different; variances were not.